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LABORATORY METHODS

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The Effect of Temperature and Oligonucleotide Primer Length on the Specificity and Efficiency of Amplification by the Polymerase Chain Reaction

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ABSTRACT

The polymerase chain reaction (PCR) is most effectively performed using a thermostable DNA polymerase such as that isolated from *Thermus aquaticus*. Since temperature and oligonucleotide length are known to control the specificity of oligonucleotide hybridization, we have investigated the effect of oligonucleotide length, base composition, and the annealing temperature on the specificity and efficiency of amplification by the PCR. Generally, the specificity of PCR is controlled by the length of the oligonucleotide and/or the temperature of annealing of the primer to the template. An empirical relationship between oligonucleotide length and ability to support amplification was determined. This relationship allows for the design of specific oligonucleotide primers. A model is proposed which helps explain the observed dependence of PCR on annealing temperature and length of the primer.

INTRODUCTION

ENZYMATIC AMPLIFICATION OF DNA by sequential rounds of primer extension has become a useful tool for the analysis of defined DNA sequences (Kleppe *et al.*, 1971; Saiki *et al.*, 1985; Scharf *et al.*, 1986). This polymerase chain reaction (PCR) utilizes two oligonucleotide primers that hybridize to opposing strands of DNA at positions spanning a sequence of interest and a DNA polymerase for sequential rounds of template-dependent synthesis of the DNA sequence. With the introduction of a thermostable DNA polymerase from *Thermus aquaticus* (Saiki *et al.*, 1988b) PCR has been automated. It is possible to amplify specific DNA sequences exponentially so that 10^5 - to 10^7 -fold amplification can be achieved in 20-25 rounds of amplification.

PCR has been combined with either restriction endonuclease cleavage or allele-specific oligonucleotide hybridization to analyze or diagnose a number of genetic diseases (Lee *et al.*, 1987; Chan *et al.*, 1988; Saiki *et al.*, 1988a;

Hunkapiller and Hood, 1989). Enrichment of DNA sequences by PCR greatly improves the sensitivity of these diagnostic methods, and therefore PCR overcomes the primary limitation of these techniques. PCR has been used for the amplification of target DNA templates for subsequent DNA sequencing (Gyllenstein and Erlich, 1988); well as combined with a ligation based analysis of single nucleotide variation (Landegren *et al.*, 1988; Wu and Wallace, 1989). Recently, we described a method that uses PCR directly as a diagnostic method for the identification of single base-pair variations in the human genome (Wu *et al.*, 1989a,b). We have referred to this method as allele specific PCR (AS-PCR). This technique relies upon the ability (under certain conditions) of *T. aquaticus* DNA polymerase to prime DNA synthesis when a single mismatch exists at the 3' position of one primer. When performed under appropriate reaction conditions including the appropriate reaction temperature, AS-PCR provides for the direct identification of alleles by visualization of the amplified DNA fragments on an ethidium bromide-stained gel.

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A number of variables have been identified that influence the optimization of PCR (Saiki *et al.*, 1988b). During the development of AS-PCR, we became keenly aware of the importance of primer annealing temperature in PCR. To optimize the amplification (*i.e.*, maximize signal and reduce nonspecific amplification products), reactions were often performed at surprisingly high primer annealing temperatures. In most cases, the optimal primer annealing temperature exceeds the empirical dissociation temperature of the oligonucleotide (Suggs *et al.*, 1981). This observation suggests that PCR priming itself is governed by kinetic parameters and not thermodynamic parameters.

Despite the now popular use of PCR in disease diagnosis, pathogen screening (Buchbinder *et al.*, 1988; Murakawa *et al.*, 1988; Kwok *et al.*, 1989), and DNA-based allele typing (Horn *et al.*, 1988), little is known about the kinetics and other parameters governing PCR. Conditions are determined largely by trial and error. Primer design and annealing temperature choice are often somewhat arbitrary. In the present report, we present a theoretical and experimental analysis of oligonucleotide priming in PCR. We show that within a limited range of oligonucleotide primer length, the optimal PCR primer annealing temperature can be predicted from an empirical mathematic expression. Furthermore, we argue that the design of oligonucleotide primers should not be done on a totally arbitrary basis, but rather with the knowledge of the effects of length and DNA sequence on the process of amplification.

MATERIALS AND METHODS

Oligonucleotide synthesis

Oligonucleotides were synthesized on an Applied Biosystem 380B or a Cruachem PS250 DNA synthesizer using

phosphoramidite chemistry. They were purified on a 7 M urea 12% polyacrylamide gel followed by high-performance liquid chromatography as described (Miyada and Wallace, 1987).

Isolation of human DNA

Human DNA samples were isolated from peripheral blood leukocytes. DNA isolation was performed according to a modified procedure using Triton X-100 followed by Proteinase K and RNase treatment (Bell *et al.*, 1981). The average yield of genomic DNA per milliliter of blood sample was approximately 25 μ g.

Polymerase chain reaction

PCRs were carried out with multiple sets of oligonucleotide primers. These primers ranged from 14 to 20 nucleotides in length. The primers were used to amplify both unique sequences as well as variable number of tandem repeat (VNTR) regions in the human genome (see Table 1). PCRs were performed in a volume of 50 μ l containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 0.01% (wt/vol) gelatin, template DNA (5 μ g/ml), and 0.1 mM each dATP, dCTP, dGTP, and TTP (Pharmacia) with 2.5 units of *T. aquaticus* DNA polymerase (Perkin Elmer-Cetus) and 5 pmoles of each oligonucleotide primer. Following denaturation of the DNA at 95°C for 3.5 min, the amplification of the different fragments was carried out for 25 cycles as follows: annealing at the specified temperature for 2 min, polymerization at 72°C for 3 min, and denaturation at 94°C for 1 min using a Perkin Elmer-Cetus DNA thermal cycler. At the end of 25 cycles, the samples were held at 4°C in the thermal cycler until removed for further analysis.

TABLE 1. RELATIONSHIP BETWEEN PRIMER LENGTH AND SEQUENCE AND ITS ABILITY TO PRIME IN PCR

Gene	Name	Sequence	G+C/L	L _n	T _p	T _n	T _m	Ref.
β-globin	BGP-1	GGGCTGGGCATAAAAAGTCA	10/19					(Chehab <i>et al.</i> , 1987)
	BGP-2	AATAGACCAATAGGCAGAG	8/19	27	62	67	55	
H-Ras	H-Ras 5'	CTGTAGGAGGACCCCGGG	13/18					(Capon <i>et al.</i> , 1983)
	H-Ras 3'	CTCTCATGCCCTCATGCC	12/19	31	67	69		
β-globin	BGP-1	GGGCTGGGCATAAAAAGTCA	10/19					(Chehab <i>et al.</i> , 1987)
	ON14A	CACCTGACTCCTGA	8/14	22	55	59	50	
HLA DQα	HLA I	GAAGACATTGTGGCTGACCA	10/20	30	65	69		(Gyllenstein and Erlich, 1988)
	HLA F	ATTGGTAGCAGCGGTAGAGTT	10/21					
HLA DQα	DQα3 5'	ATGGTCCCTCTGGG	9/14					(Gyllenstein and Erlich, 1988)
	DQα3 3'	GAGCGTTTAATCAC	6/14	20	51	55		
33.6	33.6 5'	TGTGAGTAGAGGAGACCTCA	10/20					(Jeffreys <i>et al.</i> , 1985)
	33.6 3'	AACGTCCTGGACAGACAAAGA	9/20	29	64	67		
Insulin	INS 5'	TAAAGGCAGGGTGGGAAGTAC	11/20					(Bell <i>et al.</i> , 1982)
	INS 3'	GCCACTTTCCACATTAGACC	10/20	30	64	67		
33.4	33.4 5'	ATGGGGGACCGGGCCAGACC	15/20					(Jeffreys <i>et al.</i> , 1985)
	33.4 3'	CCAGGAGGCCACAGAACCT	13/20	33	72	74	68	
hGH	GHPCR1	TTCCCAACCATTCCTTA	8/18					(Selden <i>et al.</i> , 1986)
	GHPCR2	GGATTTCGTGTGTGTTTC	7/18	25	58	61		

L=length; L_n=normalized length; T_p=maximum temperature at which efficient PCR amplification is observed; T_n=minimum temperature at which PCR amplification is not observed; T_m=measured in 50 mM KCl, 10 mM Tris-HCl (pH 8.3); 1.5 mM MgCl₂ at 0.1 μ M duplex.

Analysis of PCR products

An aliquot (15 μ l) of the PCR-enriched DNA samples were subjected to electrophoresis in a 1.5% agarose gel. Electrophoresis was performed in 89 mM Tris-HCl, 89 mM borate, and 2 mM EDTA buffer for 3 hr at 120 V. At the completion of the electrophoresis, the gel was stained in ethidium bromide (1.0 μ g/ml) for 15 min and destained in water for 10 min at room temperature. The amplified PCR fragments were seen by UV transillumination and photographed.

Thermal denaturation

Oligonucleotides complementary to BGP-2, 33.4 3', and ON14A (Table 1) were synthesized. Duplexes were formed by mixing equimolar amounts of the complementary oligonucleotides. The solutions containing the different duplexes were adjusted to 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, a buffer which is identical to PCR buffer without the dNTPs and gelatin. Thermal denaturation was performed using a Gilford model 2527 thermo-programmer. The heating rate was 1°C/min. Absorbance was recorded on a Beckman Model 25 spectrophotometer set to single beam mode.

RESULTS

Effect of annealing temperature on the specificity and efficiency of PCR amplification

A number of factors influence the specificity of *T. aquaticus* DNA polymerase-mediated amplification: time of the primer extension step, amount of enzyme used, the concentration of cations, the nature of the template DNA and primers, and the annealing temperature (Mullis *et al.*, 1986; Kim and Smithies, 1988; Saiki *et al.*, 1988b). A significant improvement in specificity is typically obtained when the primer annealing temperature is raised gradually (Fig. 1). For most unique genomic sequences, primer-directed amplification becomes optimal when the specific fragment becomes the major amplification product. The optimal primer annealing temperature usually occurs over a 4–10°C range. Should the primer annealing temperature be raised beyond a certain temperature range, the efficiency of the amplification decreases, leading to little or no amplification of the fragment. The effect of primer annealing temperature is demonstrated in the amplification of a 456-bp fragment of the human growth hormone gene (Fig. 1). Only the specific PCR product is detected when 55–58°C is used as the annealing temperature. At primer annealing temperatures below 55°C, multiple nonspecific fragments are seen in the ethidium bromide-stained gel, indicating that the primers annealed to sites on the genomic DNA template other than the specific primer annealing sites. Above 58°C there is poor amplification efficiency.

Melting temperature of PCR primers

To determine how PCR priming efficiency of oligonucleotide primers compared with their melting temperature

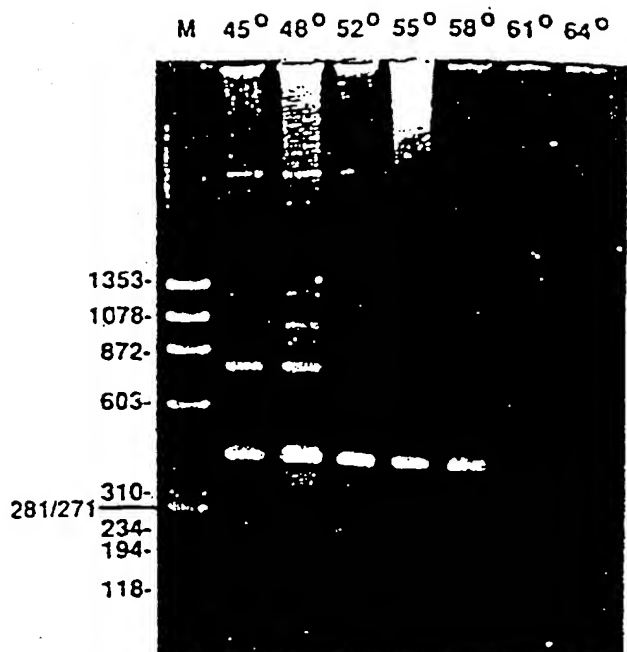


FIG. 1. Effect of temperature on the efficiency of PCR amplification. DNA (1 μ g) was amplified in a Perkin Elmer-Cetus thermocycler in a 50- μ l reaction containing 0.1 μ M human growth hormone primers (Selden *et al.*, 1986) (5'-TTCCCAACCATTCCTTA and 5'-GGATTTC-TGTTGTGTTTC) and 2.5 units of *T. aquaticus* DNA polymerase in the buffer recommended by the manufacturer of the enzyme. Various primer annealing temperatures were used; the temperature program used on the thermocycler was as follows: 3.5 min at 95°C followed by 25 cycles of 2 min at the indicated annealing temperature, 3 min at 72°C, 1 min at 94°C each and finally 1 min at the indicated annealing temperature followed by 4 min at 72°C. The products of the amplification reaction were subjected to electrophoresis on a 1.5% agarose gel in 1 \times TBE, stained with ethidium bromide and photographed under ultraviolet light. Marker is ϕ X174 RF DNA digested with *Hae* III. Numbers to the left are sizes of the marker restriction fragments in base pairs.

in PCR buffer at the concentration of oligonucleotide used in the PCR reaction; oligonucleotides complementary to three of the PCR primers described in Table 1 were synthesized. Equimolar amounts of each of the two complementary oligonucleotides were combined in PCR buffer; then the solution was heated and then slowly cooled. The duplexes formed were then subjected to thermal denaturation. The T_m values were determined from the first derivative of the melting curves and are presented in Table 1.

Relationship between effective priming temperature and effective primer length

The effective priming temperature or T_p is defined as the highest temperature at which optimal primer directed amplification occurs. As is the case for the empirical dissociation, temperature previously described for oligonucleo-

tide-DNA hybridization (Wallace *et al.*, 1979; Suggs *et al.*, 1981), T_p is linearly related to the effective length of the oligonucleotide primer (L_n) over a limited length range. L_n takes into account the greater stability of a G-C base pair compared with an A-T base pair and assumes that a G-C base pair is twice as stable as an A-T base pair. Therefore, for a given primer $L_n = 2$ [no. of G or C] + [no. of A or T]. For example, a 20-nucleotide-long oligonucleotide primer with 10 Gs or Cs has an L_n of 30. Figure 2 depicts the linear relationship between T_p and L_n over an L_n range of 20–35 (the data are tabulated in Table 1). Linear regression analysis of the data showed a linear correlation coefficient of 0.986. The straight line is defined by the equation $T_p = 22 + 1.46(L_n)$. In cases where the L_n values of the two primers are different, the smaller of the two is plotted.

DISCUSSION

The empirical linear relationship between T_p and L_n was not predicted. It nevertheless provides a practical rule for determining the optimal primer annealing temperature to be used for PCR amplification with a given set of primers. We attempted to correct for the effect of base composition or actual DNA sequence using other known relationships (Gotoh and Tagashira, 1981) but did not find a method that provides as good a correlation as that seen with the simple assumption made here (not shown). In general, the primer with the smallest L_n determines the annealing temperature unless sequence variability exist in either primer annealing region. The linear equation, $T_p = 22 +$

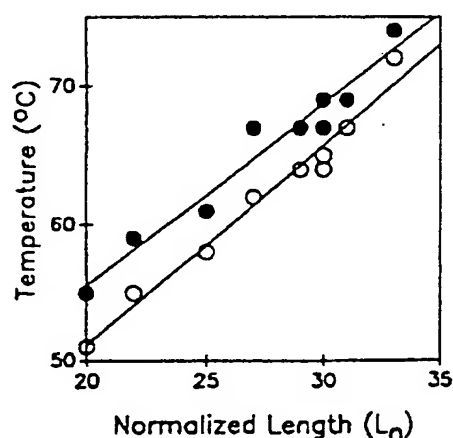


FIG. 2. Effect of temperature on the ability or inability of oligonucleotide primers of various lengths and base compositions to support amplification in the PCR. Oligonucleotide primer pairs for various genes were designed (Table 1). The primers of various sequence and length were tested for their ability to amplify the appropriate gene segment at different annealing temperatures. The maximum temperature where amplification was observed (O) and the minimum temperature where amplification was not observed (●) is plotted against the normalized length ($L_n = 2 \cdot [\text{\#G or C}] + [\text{\#A or T}]$). The lower line represents a least-squares fit through the data. The equation defining the line is $T_p = 22 + 1.46(L_n)$ with a correlation coefficient of 0.987.

$1.46(L_n)$, defines the optimal annealing temperature with optimal amplification occurring at 2–5°C above or below this calculated temperature.

Kim and Smithies (1988) have described optimized conditions for nine pairs of 19- and 20-nucleotide-long primers. The temperatures used by these authors for primers of varying G/C content are consistent with the empirical formula presented above. Rychlik *et al.* (1990) also describe an empirical approach for optimizing the primer annealing temperature. They determined the temperature that resulted in a maximal yield of product rather than the maximal annealing temperature at which amplification was observed, as was done in this report. In general the temperatures determined by the approach of Rychlik *et al.* (1990) are lower than those determined here.

PCR is controlled by the rate of primer template annealing

As we have proposed previously (Wu *et al.*, 1989a,b), the successful priming of an oligonucleotide on a DNA template is governed by two variables: The rate of primer dissociation from the primer-template complex before initiating polymerization and the rate at which the DNA polymerase extends the primer until a stable primer-template complex is formed. PCR is governed kinetically; once a transient association between primer and template DNA has occurred, the addition of the first few nucleotides to the primer forms a stable primer DNA complex, thereby allowing the continued extension of the primer until the product is complete on the template.

The above relationship explains why the optimal temperature of priming is higher than the T_m of the primer-DNA duplex. Within the limited range of L_n examined, T_p for primers exceeds the melting temperature by an average of 5–10°C (Table 1 and Fig. 2). The melting temperature of oligonucleotides, T_m , describes the temperature at which 50% of the oligonucleotide duplex dissociates under particular concentrations of duplex and cation. Although the temperature T_p is greater than T_m , where the primers are not expected to be annealed stably to the template, priming occurs by the elongation of the primer when it interacts transiently with the template at the annealing site. Unlike oligonucleotide hybridization, which is governed by equilibrium, primer annealing in PCR depends upon the balance of the rates of primer dissociation and elongation.

Extrapolation of T_p data in Fig. 2 shows that as L_n increases, the priming temperature will eventually exceed the optimal temperature for elongation by the polymerase. This temperature, 74°C for *T. aquaticus* DNA polymerase (Saiki *et al.*, 1988b), represents the T_p for an oligonucleotide primer with L_n of 38. This primer length should probably be considered the maximum L_n to be used in designing a PCR primer. Using primer with L_n greater than 38 would require temperatures greater than the optimal polymerization temperature (74°C) to achieve optimal specificity. It should be noted that the arbitrary choice of primer annealing temperature (e.g., the popular use of 37°C) should be avoided if specificity is the object of the amplification reaction. Finally, since the temperature T_p is

the maximum temperature at which priming occurs with a given oligonucleotide pair, this is also the temperature at which maximum specificity is achieved. This is especially important in the case of AS-PCR where one can discriminate between two DNA sequences that differ by a single nucleotide.

In some PCR applications, Mg^{2+} has been adjusted to optimize the PCR reaction. We have tested the effect of different Mg^{2+} concentrations on the T_p value and have found only minor affects. The experiments described in Figs. 1 and 2 and in Table 1 were performed at 1.5 mM Mg^{2+} . At higher Mg^{2+} concentrations, the temperature of effective priming was elevated only slightly (e.g., by 3°C at 10 mM Mg^{2+}).

In summary, whether a primer is to be extended or not depends on which event will predominate—primer dissociation or primer elongation. At higher temperatures, shorter oligonucleotides will dissociate more rapidly compared to longer oligonucleotides.

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